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(54) Title: HUMAN ERYTHROPOIETIN cDNA CLONES (57) Abstract A peptide immunochemically reactive with a monoclonal antibody to human erythropoietin, to a DNA fragment with a sequence coding for such peptide, to a recombinant DNA molecule having said DNA fragment inserted therein and to a transformed organism comprising an expression vector having said DNA molecule inserted therein, said organism being capable of producing said peptide, either directly or as a fusion protein. The present invention is also directed to methods for producing each of the above peptide, DNA fragment, DNA molecule and transformed organism.		

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HUMAN ERYTHROPOIETIN cDNA CLONES

The United States Government has rights to this invention by virtue of grants No. RO1-HL21683 and No. RO1-HL30862 by the National Institute of Health, Bethesda, Maryland.

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Field of the Invention

The present invention relates to cDNA clones of human erythropoietin (Ep), to methods of identification and preparation of such clones, and to their expression products. More particularly, the present invention relates to:

20

(a) cDNA clones of human Ep identified from a cDNA-library constructed from human kidney mRNA, (b) synthesis of human Ep cDNA and insertion into pBR322 plasmids, (c) generation of E.coli expressing said cDNA, and (d) the products of expression of said cDNA.

25

The entire disclosure of my copending United States patent application Serial No. 570,075, filed on January 11, 1984 and entitled "REVERSE IMMUNOAFFINITY CHROMATOGRAPHY PURIFICATION METHOD" is hereby incorporated by reference as if fully set forth herein (said application being hereinafter referred to as the "Ep Purification Patent Application").

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The entire disclosure of my copending United States patent application Serial No. 570,039, filed on January 11, 1984 and entitled "MONOCLONAL ANTIBODY TO HUMAN URINARY ERYTHROPOIETIN AND HYBRIDOMA SECRETING SAID ANTIBODY" is hereby incorporated by reference as if fully set forth herein (said application

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being hereinafter referred to as the "Anti-Ep Patent Application"). Copies of extensive excerpts from both applications are attached as Appendix A and B, respectively.

Background of the Invention

A reliable and plentiful supply of human Ep has long been in demand to assist in a better understanding of the molecular mechanism of erythropoiesis and thus contribute to (a) the development of methods for the diagnosis and treatment of anemias and (b) the understanding of the differentiation and development of mammalian cells. Unavailability of sufficient human Ep has been due to scarcity of raw material, difficulties in its purification and lack of precise knowledge on the biogenesis of Ep.

The progress made by the present inventor in native human Ep purification (described in the Ep Purification Patent Application) by direct and reverse immunoaffinity chromatography, and in preparation of monoclonal Anti-Ep (described in the Anti-Ep Patent Application) has made it possible to attempt cloning of human Ep genes which, upon expression, can produce Ep protein.

Objects of the Invention

Accordingly, it is an object of this invention to provide an alternative source of human Ep protein.

Another object of this invention is to identify human Ep mRNA.

Yet another object of this invention is to synthesize and characterize human Ep cDNA.

Still another object of this invention is to produce a recombinant organism expressing human Ep cDNA.

A further object of this invention is to purify the product of said Ep cDNA expression.

A still further object of this invention is to characterize the sequence of human Ep cDNA and to identify the composition of human Ep.

These and other objects of this invention will be apparent to those skilled in the art in light of the present description, accompanying claims and appended drawings.

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Summary of the Invention

One aspect of the present invention relates to a peptide immunochemically reactive with a monoclonal antibody to human erythropoietin.

Another aspect of the present invention relates to a DNA fragment coding for said peptide.

Yet another aspect of the present invention relates to a DNA molecule having inserted therein a DNA fragment comprising a DNA sequence coding for said peptide.

Still another aspect of this invention relates to a transformed living organism containing said DNA molecule said organism being capable of expressing said peptide.

Finally, this invention relates to methods for producing the above peptide, DNA fragment, DNA molecule and organism.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is a fluorograph of electrophoresis of in vitro translation products of human kidney poly(A)⁺ mRNA and immunoprecipitation of the translation products by monoclonal Anti-Ep.

Figure 2A depicts a silver stain of total proteins of human kidney extract, crude and pure Ep resolved by SDS-PAGE.

Figure 2B depicts an autoradiograph of an immunoblot from identical samples showing immunological detection of Ep-specific proteins.

Figure 3 is an autoradiograph of electrophoresis of the in vitro translation products of agarose gel size-fractionated mRNA.

Figure 4A is an autoradiograph of a nitrocellulose filter bearing positive colonies, as detected by colony hybridization.

Figure 4B is an autoradiograph of the same type of filter bearing a positive clone, pEp2, expressed in E.coli as a beta-lactamase fusion protein, detected by in situ radioimmunoassay using monoclonal Anti-Ep.

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1 Figure 5 is a photograph of agarose gel electrophoresis of cDNA inserts for positive Ep clones, visualized by ethidium bromide staining and ultraviolet transillumination.

5 Figure 6A is a fluorograph of electrophoresis of in vitro translation products from mRNA selected by hybridization with positive Ep clones.

 Figure 6B is an Anti-Ep immunoblot of electrophoresis of in vitro translation products of mRNA selected by hybridization with positive Ep clones.

10 Figure 7 is an autoradiograph of competitive immunoprecipitation of hybrid selected translation products of M.W. 29,000 and 15,000 in the absence or presence of unlabeled Ep.

Detailed Description of the Invention

15 In order to clone Ep cDNA, its functional mRNA is first isolated. Difficulties in isolating this mRNA stem from the fact that the specific cellular site of Ep synthesis has not been firmly established, although the kidney is known to play a key role. Additional difficulties arise
20 from the scarcity of viable human kidney samples and from their low Ep levels. It was therefore necessary to search for sources with elevated Ep levels and greater tissue availability.

 It has been well documented that erythrocytosis
25 may be associated with various renal tumors. Tumor extracts from some renal carcinoma patients have shown an increased level of Ep and such tumors were often considered as Ep-producing tumors. However, renal carcinoma tumors are only rarely accompanied by significantly increased Ep
30 levels. Thus, the ability of renal carcinoma tissue to produce Ep after explantation (and in culture) is by no means assured. Accordingly, the present inventor engaged in an extensive search for human renal tumors showing increased Ep levels, in an effort to identify and secure a
35 continuous source of functional Ep mRNA.

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1 In the work leading to the present invention, a
total of thirty-six (36) renal cell carcinomas were exam-
ined. Two of those demonstrated very high levels of Ep
(0.9 and 3 units/g of tissue) by exhypoxic polycythemic
5 mouse bioassay. It was observed that both normal tissue
and cancerous tissue explants of carcinoma-affected kidneys
sometimes displayed elevated Ep titers. By contrast,
tissue explants of normal kidneys showed no detectable Ep
activity by the same assay.

10 Prior to the present invention, there had been no
specific procedure for the isolation of human kidney mRNA.
Renal tissue is very rich in mRNA-inactivating ribonu-
cleases (RNase) and is extremely difficult to homogenize.
Further difficulties arise because of the limited availabil-
15 ity of nephrectomy samples. Accordingly, conventional
fractionation of tissue samples (into cytoplasmic, micro-
somal and nuclear fractions) is impractical. Thus, total
cellular RNA is preferably first isolated by extraction of
disrupted kidney cells and messenger RNA is then selective-
20 ly enriched, as follows:

Both normal and tumor portions from Ep-positive
samples of renal carcinoma-affected tissues should be used
for mRNA preparation. Throughout the procedure, care must
be taken to minimize the degradative effect of (endogenous
25 or exogenous) ribonuclease. Thus, preferably, RNase
inhibitors are introduced in the extraction buffer.
Reagents are sterilized and the glassware is baked at 250°C
overnight prior to contact with RNA samples. Vanadyl-
ribonucleoside (VRNS) complex or RNasin are preferred
30 exogenous inhibitors of RNase, with VRNS being most pre-
ferred. Preferably, the extraction buffer also includes a
combination of chaotropic agents (such as ethylene glycol,
propylene glycol or other common glycols) or dissociating
agents (e.g., sarkosyl, guanidine hydrochloride and guan-
idine isothiocyanate) and reducing agents (such as beta-
35 mercaptoethanol). In general, the methods and reagents
used significantly affect the yield and functionality of

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1 the mRNA obtained. Most preferably, the extraction buffer
contains guanidine isothiocyanate, sarkosyl, beta-mercapto-
ethanol, sodium citrate and vanadyl-ribonucleoside complex,
5 which serve to disintegrate cellular structures, disso-
ciate proteins and inactivate degradative enzymes and
RNases.

The method of mRNA isolation employed in the
present invention is fast, convenient and results in a good
yield. The initial cell disruption is very important and
10 it is preferably accomplished by dry-blending the tissue
(as in an ordinary blender) in the presence of liquid
nitrogen to ensure a sufficiently low temperature at which
all enzymes will be inactive.

The powdered tissue is then treated with the
15 extraction buffer. This is most conveniently and thorough-
ly accomplished in the powdering blender. Dissolution of
the cellular material and shearing of the DNA takes place
by further blending, and passing the sample through a
needle. Shearing is evidenced by a significant drop in
20 viscosity of the mixture.

The general procedure of Ulrich et al: Rat
Insulin Gene: Construction of Plasmid Containing the Coding
Sequences, Science 196: 1313 (1977), was used with specific
modifications as described in Example 2.

25 The concentration of total RNA is measured by
absorbance at 260nm. The absorbance ratios 260/230 and
260/280 are close to 2. This indicates no
contamination of proteins and carbohydrates. A yield of
about 0.5 to 1mg of RNA/gram of tissue is obtained.

30 Polyadenylated RNA (poly(A)⁺ RNA) is then
selected on an oligothymidylic acid-cellulose (oligo-dT
cellulose) column as described by Aviv, H. et al, Purifica-
tion of Biologically Active Globin Messenger RNA by Chroma-
tography on Oligothymidylic Acid-Cellulose, Proc. Nat.
35 Acad. Sci. (USA) 69:1408-1412 (1972). The poly(A)⁺ RNA
thus selected can be stored in ethanol at low temperature.
Under these conditions, stability of the RNA is insured.

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1 In order to confirm that the poly(A)⁺ RNA
contains Ep message (functional Ep mRNA) some of the oligo
dT-cellulose-selected mRNA is translated in vitro. Trans-
lation may take place in a wheat germ cell-free system or,
5 preferably, in a mRNA-dependent rabbit reticulocyte lysate
system, as described by Pelham, H.R.B. et al, An Efficient
mRNA-Dependent Translation System from Reticulocyte Lysates.
Eur. J. Biochem. 67:247-256 (1976). The presence of mRNA
coding for Ep protein in the poly(A)⁺ kidney RNA is
10 confirmed by immunoprecipitation of the translation
products. Immunoprecipitation is carried out according to
Kessler, S.W. Use of Protein-A Bearing Staphylococci for
the Immune Precipitation and Isolation of Antigens from
Cells: Meth. Enzymol. 73:442, 1981, with purified mono-
15 clonal Anti-Ep IgG designated as 7A7. This specific
procedure is described in Example 4. Two polypeptides are
immunoprecipitated with monoclonal Anti-Ep. Their molecu-
lar weights are 29,000 and 15,000 daltons, respectively
(Fig. 1, lane 2). These polypeptides are not precipitated
20 by preimmune mouse serum (Fig. 1, lane 3) nor are they
detected in endogeneous translation and its immunopreci-
pitated sample (Fig.1, lanes 6 and 7). The size of these
polypeptides is smaller than expected for native Ep.
However, in a rabbit reticulocyte lysate system, where the
25 ribosomes are free in the cytosol and where endoplasmic
reticulum membrane organization, and membrane bound enzymes
are absent, post-translational modification such as glyco-
sylation is not expected to occur. Thus, the 29,000 dalton
polypeptide which is precipitated specifically by Anti-Ep,
30 may represent the aglycosylated form of Ep. The 15,000
dalton peptide may represent an immunologically related
species. The presence of these polypeptides in tissue is
also confirmed by immunoblotting of the tissue extract
along with authentic Ep (Figure 2). The details of these
35 experiments are described in Example 4.

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1 Some of the thus obtained mRNA is enriched in Ep
mRNA by size fractionation on agarose gel in the presence
of CH_3HgOH according to Bailey, J.M. et al, Methylmercury
5 as a Reversible Denaturing Agent for Agarose Gel Electro-
phoresis, Anal. Biochem. 70:75 (1976). The resolved RNA
fractions are eluted and samples thereof are translated.
The translation product is tested for Ep presence, prefer-
ably by immunoprecipitation with monoclonal Anti-Ep ac-
cording to the method of Kessler, supra.

10 The fraction of agarose-gel-fractionated RNA that
was enriched in Ep mRNA (fraction 11 in Fig. 3) is reverse-
transcribed into [^{32}P]-labeled cDNA which is used as a
hybridization probe in the identification of cDNA positive
clones. Reverse transcription (using Avian myeloblastosis
15 virus (AMV) reverse transcriptase) is preferable to T4
kinase RNA phosphorylation treatment because it gives a
higher specific radioactivity (of the order of 10^7
cpm/microgram) and it is less likely to label single-
stranded contaminating ribosomal or tRNAs.

20 The remainder of poly(A) $^+$ kidney mRNA is used
as the template for the synthesis of the 1st strand of cDNA
by AMV reverse transcriptase in the presence of oligo-
dT₍₁₂₋₁₈₎ as the primer. In view of: (a) the very rare
opportunity presented in this case, i.e. the availability
25 of a human kidney sample with significantly elevated Ep
activity. and (b) the difficulties encountered in the
preparation of intact polysomes from RNase-rich renal
tissues, construction of a total human kidney cDNA library
was undertaken. This approach not only entails minimum
30 handling of the limited amounts of valuable RNA, but also
allows other renal proteins of medical interest to be
screened. Such a cDNA library would, of course, also
require the screening of a large number of recombinants
for Ep clones.

35 Conditions optimal for reverse transcription are
highly dependent on the particular type of mRNA. The

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1 purity of the reverse transcriptase, its RNase contamination,
the ratio of reverse transcriptase activity to mRNA
quantity, the substrate concentrations, the pH and ionic
5 conditions of the reaction mixtures are all important
factors which influence transcription efficiencies.

A ratio of 6 units of reverse transcriptase per
microgram of mRNA template is preferred. Vanadyl ribo-
nucleoside complex is preferably added to the transcription
reaction mixture to inactivate contaminating RNase. The
10 most preferred reaction conditions are set forth in Example
5. The general reverse transcription procedure is conducted
according to Retzel, E.F. et al., Enzymatic Synthesis
of DNA by the Avian Retrovirus Reverse Transcriptase in
vitro, Biochem. 19:513-518, 1980.

15 At the end of the reaction period, template RNA
is denatured preferably with CH_3HgOH to avoid interference
of template mRNA in the synthesis of ds-cDNA.

Complementary cDNA is preferably synthesized
using the large (Klenow) fragment of E.coli DNA polymerase
20 I (which does not have the 5' \rightarrow 3' exonuclease activity)
according to the method of Efstratiadis, A., et al.,
Enzymatic In Vitro Synthesis of Globin Genes, Cell, 7:279,
1976, wherein the reverse transcriptase loop from the
previous step serves as the primer.

25 The hairpin loop covalently linking the first and
second strand of cDNA is cleaved by nuclease S_1 . The
amount of S_1 required is determined by titration in pilot
experiments using alkaline agarose gel electrophoresis,
which permits the sizes of the DNA molecules to be visual-
30 ized. The Klenow product behaves as a molecule twice the
size of cDNA, and the blunt ended double stranded cDNA (ds
cDNA) behaves as two molecules the size of the original
cDNA strand. The optimum amount of S_1 nuclease was found
to be 2.5 units per microgram of ds cDNA in this case.

35 In this work, 60 micrograms of poly(A)⁺ RNA
yielded 5 micrograms of blunt-ended ds cDNA.

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1 The ds cDNA is inserted in the Pst I restriction
endonuclease site (located in the ampicillin resistance
gene) of pBR322 by homopolymeric dC:dG tailing according to
Villa-Komaroff et al., A Bacterial Clone Synthesizing
5 Proinsulin, Proc. Nat. Acad. Sci. (USA) 72:3727, 1978.
The oligo(dC)-tailed cDNA and oligo(dG)-tailed vector are
annealed preferably at a ratio of 1:2.

pBR322 is preferred because of its versatility as
a plasmid cloning vector. It is under relaxed control and
10 contains both ampicillin and tetracycline resistance genes.
The Pst I cleavage site is a single cleavage site within
the ampicillin resistance gene. Cloning into the Pst I
site inactivates this gene. Thus, the clones will be
ampicillin-sensitive. In addition, they should also be
15 tetracycline-resistant, if they have taken up the plasmid.

The recombinant plasmids are transformed into
E. coli C600 made competent to take up DNA by CaCl_2 and
heat shock as described by Mandel, M. et al. Calcium
20 Dependent Bacteriophage DNA Infection, J. Mol. Biol.
53:159, 1970.

Transformants are selected for tetracycline
resistance and ampicillin sensitivity. Since, as mentioned
above, the Pst I site is in the ampicillin resistance gene,
transformants will be ampicillin-sensitive (Amp^S) but
25 tetracycline-resistant (Tet^R).

E. coli C600 is preferably used as the recipient
because it is a good host for large scale growth and
purification of plasmids and can be transformed with high
efficiency by use of a plasmid vector annealed to cDNA by
30 dC:dG homopolymeric tailing.

For maximum efficiency of transformation, the
bacterial culture should be in the logarithmic phase of
growth and the cell density about 5×10^7 cell/ml at the
time of treatment with calcium chloride. Maintaining the
35 cells on ice for 12-24 hours prior to the transformation
significantly increases transformation efficiency.

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1 The optimum transformation reaction ratios are
1-10 ng of cDNA to 100 microliters of cell suspension.
Larger amounts of suspension or cDNA result in lower
transformation efficiencies. Use of top agar as a trans-
5 formation plating medium is preferred.

 Although there are several procedures for identi-
fication of recombinant plasmids that incorporate Ep DNA,
the colonies are preferably initially screened by colony
hybridization using [³²P] labeled cDNA synthesized from
10 enriched Ep mRNA as the probe, followed by in situ colony
radioimmunoassay (RIA) using purified monoclonal Anti-Ep
IgG.

 Colony hybridization is preferably conducted
according to the general method of Grunstein, M. et al.
15 A Method for the Isolation of Cloned DNAs that Contain a
Specific Gene, Proc. Nat. Acad. Sci.(USA) 72:3961, 1975
here adapted for a smaller scale (in order to save
materials, which are in short supply, while maintaining
sensitivity of the screening procedure) by use of very
20 small amounts of [³²P]-labeled cDNA as a probe with high
specific radioactivity and small nitrocellulose filters,
thus permitting a large number of colonies to be screened
at a time. Duplicate colonies of transformants are grown
on small filters, colonies are lysed and the DNA is de-
25 natured (by alkali treatment). Cell debris are treated
with proteinase K and the DNA is fixed to the filter by
baking. The DNA is hybridized to the probe, which binds
only to its complementary DNA and permits positive colonies
to be identified by autoradiography. This procedure is
30 very efficient and results in elimination of about 95% of
the colonies from further screening.

 Positive colonies are picked and grown on nitrocel-
lulose filters for further screening by in situ colony RIA.
This method requires the expression of antigenic determin-
35 ants, i.e., the expression of the cDNA inserted in pBR322
to produce a fused polypeptide containing the appropriate

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1 antigenic site for Anti-Ep recognition. Given the avail-
ability of monoclonal Anti-Ep, RIA is a natural screening
method choice for identification of positive clones. RIA
5 is a particularly sensitive technique requiring only a
small quantity of antigen containing an antigenic deter-
minant. The general method of Helfman, D.M. et al:
Identification of Clones that Encode Chicken Tropomyosin
by Direct Immunological Screening of a cDNA Expression
Library, Proc. Nat. Acad. Sci. (USA) 80:31 (1983) was
10 employed.

Many operational variations of RIA are possible
(direct or indirect, using ^{125}I -labeled-Anti-Ep or
 ^{125}I -labeled second antibody). The binding capacity and
purity of the antibody significantly affect RIA sensitivity.
15 Thus, use of crude ascitic fluid or only partially purified
antibody should be avoided.

Radioiodination of antibodies is preferably
carried out by the lactose peroxidase enzyme beads method
as disclosed by Marchalonis, J.J., An Enzymic Method for
20 the Trace Iodination of Immunoglobulins and Other Proteins,
Biochem. J. 113:299, 1969.

In this work, three positive clones were identi-
fied and designated pEp1, 2 and 3. All three reacted
consistently with monoclonal Anti-Ep 7A7 (both ^{125}I -
25 labeled and unlabeled).

RIA-positive clones are grown in culture.
Plasmid DNAs are isolated and the size of their cDNA
inserts is determined by digestion with Pst I restriction
endonuclease followed by electrophoresis in 6% polyacryl-
30 amide. Phage ϕX174 RF DNA Hae III digested fragments are
used as size markers. The sizes of the inserts are ap-
proximately 1400, 600 and 200 base pairs (bp) for pEp1, 2,
and 3 respectively.

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1 To further confirm that the positive clones indeed contain Ep sequences, hybridization selection of Ep-specific mRNA is performed as disclosed by Parnes, et al.

5 Mouse beta-2 Microglobulin cDNA Clones: A Screening

Procedure for cDNA Clones Corresponding to Rare mRNAs,

Proc. Nat. Acad. Sci. (USA) 78:2253, 1981, modified as follows: Plasmid DNA is suspended in H₂O at 1mg/ml, heat denatured in 0.25 N NaOH, neutralized and spotted on nitrocellulose. The filters containing DNA are hybridized with poly(A)⁺ RNA. It is advisable to use relatively large amounts of mRNA for clear results. (2.5 micrograms poly(A)⁺ RNA/microgram of plasmid DNA).

Hybrid-selected mRNA is eluted from the filters, and translated in vitro as described above. ³⁵S-labeled

15 translation products are analyzed by SDS-PAGE and fluorography. All three clones hybrid selected mRNA which directed the synthesis of polypeptides of molecular weight 15,000, 29,000, 66,000 and possibly 92,000 daltons.

Final confirmation of the identity of the polypeptides was made by immunoblotting and competitive immunoprecipitation.

20 SDS-PAGE of in vitro translation products from hybrid-selected mRNA are electrophoretically transferred onto a sheet of nitrocellulose paper (0.45 micron pore size) for immunoblotting with purified monoclonal Anti-Ep

25 7A7 IgG (obtained as described in the Anti-Ep patent application). This procedure, disclosed by Towbin, H. et al:

Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Nitrocellulose Sheet: Procedure and Some

Applications, Proc. Nat. Acad. Sci. (USA) 76:4350 (1979),

30 is described in detail in Example 9. Competitive immunoprecipitation is carried out in the presence of excess unlabeled pure Ep as given in Example 10. Of the hybrid selected in vitro synthesized polypeptides, the 29,000

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1 dalton and 15,000 dalton polypeptides react with Anti-Ep on immunoblots and by immunoprecipitation and compete with authentic Ep for antibody binding.

5 The molecular weight of native human urinary Ep is 34,000 daltons. However, immunoblotting and immunoprecipitation of in vitro translation products of total kidney mRNA and hybrid selected mRNA all result in two polypeptides of M.W. 29,000 daltons and 15,000 daltons. Immunoprecipitation of tissue extracts from the Ep-rich renal carcinoma samples also resulted in identification of these two polypeptides, in addition to the 34,000 daltons polypeptide. The 34,000 daltons polypeptide is identical in size to the native glycosylated Ep. By contrast, the 29k polypeptide is smaller than expected for native glycosylated Ep. It is believed that in vitro translation in the message-dependent rabbit reticulocyte lysate system accounts for the smaller molecular weight, as discussed above. The hybrid selected 29,000 dalton peptide which was recognized specifically by monoclonal Anti-Ep is believed to be the aglycosylated form of Ep. The 15,000 dalton peptide, clearly immunologically related to, and sharing the antigenic determinants with, the 29,000 dalton and 34,000 dalton peptide, is believed to be an Ep-related protein.

25 Judging from the molecular weight of the native human urinary Ep, the cDNA insert of clone pEp1 is within the range of the coding size while those of clones pEp2 and pEp3 are too short to encode the complete sequence of Ep. These DNAs are useful in the preparation of full-length human Ep cDNA sequences for expression.

30 Alternatively, a cDNA fragment located at the 3' end of the mRNA, as determined by restriction mapping, will be used as a primer of mRNA reverse transcription. Since the 3' primer is a specific Ep cDNA fragment, the only cDNA synthesized by this reverse transcription reaction will be 35 Ep cDNA. Optimization of reverse transcription is expected to yield entire Ep cDNA.

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1 Sequencing will be performed according to the
techniques described in detail by Maxam, A.M. et al, A New
5 Method for Sequencing DNA, Proc. Nat. Acad. Sci. (USA)
74:560, 1977 and Sanger, F., et al., DNA Sequencing with
15 Chain Terminating Inhibitors, Proc. Nat. Acad. Sci.(USA)
74:5463 (1977).

The following examples serve to illustrate the
present invention without limiting its scope:

Materials and Sources:

10 agarose, and vanadyl-XTP's complex were from
Bethesda Research Laboratories (BRL), Bethesda, MD.

creatine phosphate, spermidine, essential amino
acids, Hepes, calf thymus DNA, lysozyme, tetracycline,
15 ampicillin, dithiothreitol, ethylenediamine triacetate
(EDTA), hemin, and sodium deoxycholate were from Sigma
Chemical Co., St. Louis, Mo.

restriction endonucleases, pBR322 plasmid, E. coli
DNA polymerase I, nuclease S₁, and phage ϕ X174
20 DNA-Hae III were from New England Biolabs, Beverly,
Mass.

nitrocellulose filters were from Millipore,
Bedford, Mass.

proteinase K, and calf liver tRNA were from
Boehringer Mannheim Biochemical, Indianapolis, Indiana.

25 avian myeloblastosis virus (AMV) reverse transcrip-
tase was from Life Sciences, Inc., St. Petersburg,
Florida.

Sephadex G100, and protein A were from Pharmacia,
Piscataway, N.J.

30 1,4-piperoxine-diethanesulfonic acid (PIPES),
deionized formamide (dF), guanidine isothiocyanate,
cesium chloride, and Sarkosyl were from Fluka Chemical
Corp., Hauppauge, N.Y.

bovine serum albumin was from Schwartz-Mann
35 Biochemical, Spring Valley, N.Y.

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1 oligo-dT primer, and oligodithymidylic cellulose
were from Collaborative Research, Waltham, Mass.

 [³⁵S]-Methionine, ¹²⁵I-goat anti-mouse IgG,
5 and lactose peroxidase enzyme beads were from New
England Nuclear Company, Boston, Mass.

 DNase was from Worthington, Freehold, N.J.
 nitrocellulose sheet were from Schleicher &
Schuell, Keene, N.H.

 Triton X-100 (TM) detergent was from Eastman
10 Kodak, Rochester, N.Y.

EXAMPLE 1:

Search for Human Renal Samples with High Ep Titer

 Over a period of two and a half years, an exten-
15 sive search was conducted for surgical renal carcinoma
samples with Ep activity. Tissue from nephrectomy was
obtained as fresh as possible, separated into normal and
tumor portions, and washed with sterile, ice-cold phosphate-
buffered saline to remove blood and extraneous material.
20 Small portions were set aside for Ep bioassay and for the
establishment of continuous culture. The remainder was
frozen quickly in liquid nitrogen and stored at -70°C for
the isolation of Ep mRNA. Ep activity in the tissue
extracts was assayed in vivo by the exhypoxic polycythemic
25 mouse method. Tissue homogenates were prepared by powder-
ing the sample in liquid nitrogen and extracting it with
20 mM sodium phosphate, pH 7.8, (equal ratio, wt(g)/vol
(ml)). The extract was then centrifuged at 30,000 x g for
30 minutes to remove cell debris and the clear supernatant
30 was used for Ep bioassay.

 Of 36 renal cell carcinoma extracts examined, two
demonstrated high Ep activity (0.9 and 3.0 Ep units/ml),

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1 six showed moderate activity (0.1 to 0.7 Ep units/ml) and
the rest had either marginal (<0.1 Ep units/ml) or unde-
tectable Ep activity. Tissue extracts of normal kidney
from autopsy samples generally showed no detectable Ep
5 activity but occasionally showed an activity of less than
0.05 units/ml. The increase in Ep activity in the renal
carcinoma samples was found in both the normal and tumor
portions of the kidney. The samples with high Ep bioactiv-
ity were used for mRNA preparation.

10
EXAMPLE 2:

Isolation and Purification Of Functional Human Kidney RNA

A nephrectomy sample from a patient suffering
from renal carcinoma with erythrocytosis and elevated Ep
15 activity (Ep positive) of 3.0 units/g of tissue, was
selected from Example 1.

Both the normal and tumor portions of the renal
tissues were (separately) used for mRNA preparation.

Total human kidney mRNA was isolated by the
20 guanidine/ cesium chloride method of Ulrich et al, supra.
RNA preparations were made from 10 g batches as needed.
The frozen tissue was powdered in liquid nitrogen in a
blender. 5 volumes of an extraction buffer were then
added. The extraction buffer contained: 6M guanidine
25 isothiocyanate/ 5mM sodium citrate pH 7.0/0.1 M beta-
mercaptoethanol/0.5% Sarkosyl. The mixture was homogenized
for an additional 3 min. DNA was sheared by passing the
mixture through a 22 gauge needle. The solution was
centrifuged at 10,000 rpm for 15 min and the supernatant
30 was collected. Cesium chloride was added to 0.4 g/ml of
solution and the resulting solution was layered onto a 5.7

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1 M CsCl cushion containing 0.1 M EDTA (pH 7.5) (3 ml cushion
and 2 ml of tissue extract) in a Beckman SW 50.1 centrifuge
tube. Centrifugation was at 25,000 rpm for 16 hr at 20°C.
The RNA pellet was dissolved in 10mM Tris-HCl, pH 7.4/5 mM
5 EDTA/1% SDS, extracted with phenol, chloroform/1-butanol
(4:1, v/v) and precipitated with ethanol.

EXAMPLE 3:

Selection of Poly(A)⁺ RNA

The total RNA of Example 2 was poly(A)⁺ selec-
10 ted on an oligo-dT cellulose column (0.9 x 10cm; bed vol
6.4 ml). RNA was suspended in 10mM Tris-HCl, 0.5M NaCl
buffer (pH 7.4) and loaded onto the column. Selectively
adsorbed poly(A)⁺ RNA was eluted with 10mM Tris-HCl, pH
7.4, containing 1mM EDTA and 0.1% SDS and precipitated with
15 2 vols of ethanol at -20°C for 24 hours. The pellet was
washed with 70% ethanol, resuspended in water and stored at
-20°C. Usually 0.5 to 1 mg of total RNA was obtained from
each gram of renal tissue and about 20 µg of poly(A)⁺
RNA was obtained per mg of total RNA.

20 EXAMPLE 4:

In Vitro Translation and Immunoprecipitation

In vitro translation was carried out in a message-
dependent rabbit reticulocyte lysate system, using [³⁵S]-
methionine as a label (New England Nuclear, 1236 Ci/mmol).
25 The lysate was preabsorbed with purified IgG of monoclonal
Anti-Ep (50 µg/ml) and cleared with fixed Staphylococcus
aureus bearing protein A (250 µg/ml, IgGSorb, obtained
from New England Enzyme Center, Boston, Mass.). The
reaction mixture (25 µl) contained 1 to 5 µg/ml
30 poly(A)⁺ RNA, [³⁵S]-methionine (NEN) 55 µCi, Hepes
(pH 7.6) 20mM, KCl 80mM, Mg(OAc)₂ 1.3 mM, and reticu-
locyte lysate 10 µl. Incubation was carried out at 37°C
for 1 hr. The labeled translation products were immunopre-
cipitated with purified IgG of monoclonal antibody to human
35 Ep. Immunoprecipitation was carried out according to

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1 Kessler, supra, using 5 to 50 μ g of IgG. The reaction
components were pre-absorbed with mouse serum and the
incubation was carried out in 10 mM Tris-HCl, pH 8.2/0.15 M
NaCl at 4°C for 24 hours. The immune complex was collected
5 by 100 to 200 μ l of a 10% suspension of fixed Staphylo-
coccus aureus bearing protein A by incubation at 4°C for 1
hour. The precipitate was washed 6 times with 50 mM
Tris-HCl, pH 7.4./0.15 M NaCl/1 mM EDTA/0.1% NaDodSO₄/1%
Na deoxycholate/1% Triton X-100 (Buffer W) and suspended in
10 gel sample buffer for electrophoretic analysis. The
translation products and their immunoprecipitated products
were resolved by SDA-PAGE and analyzed by fluorography
(Figure 1). Two immunospecific polypeptides were identi-
fied, one migrating at about MW 29,000 and the other at
15 about 15,000 daltons (lane 2). These polypeptides were not
precipitated by mouse preimmune serum (lane 3) and they
were not detected in endogeneous translation and in the
immunoprecipitated sample of such translation (lanes 6
and 7). As discussed above, the M.W. 29,000 peptide which
20 was precipitated specifically by the monoclonal Anti-Ep,
may represent the aglycosylated form of Ep. The M.W.
15,000 peptide, clearly immunologically related, may
represent a precursor or degradative fragment of the
aglycosylated Ep. To verify the existence of these poly-
25 peptides in the original tissue extract, immunoblotting was
conducted. Crude and purified human urinary Ep were also
included on the same blot so as to provide a direct
comparison of their immunospecificity. A single poly-
peptide of M.W. 34,000 was blotted from both the crude and
30 purified Ep (Figure 2B, lanes 2 and 3), whereas three
polypeptides were detected in the tissue extract, M.W.
34,000, 29,000 and 16,000 daltons. The 34,000 dalton
polypeptide is identical in size to the authentic gly-
cosylated Ep, and the smaller polypeptides are likely
35 candidates for the aglycosylated precursor or degraded
forms of Ep. The presence of these immunospecific poly-

-20-

1 peptides both in the tissue extract and in the in vitro
translation products supports their identification as
Ep-related forms and the presence of functional Ep mRNA.
An identical set of unblotted gel samples was subjected to
5 silver stain for analysis of their total proteins (Figure
2A), with respect to their Ep-specific proteins (Figure
2B). The immunospecificity of monoclonal 7A7 is self-
evident. These results indicate that the monoclonal
Anti-Ep recognizes native glycosylated Ep as well as
10 aglycosylated Ep and its precursors and fragments.

The monoclonal antibody to human Ep used in this
work was prepared as described in detail in the Anti-Ep
patent application.

Purified IgG was used for all immunoprecipita-
15 tion, immunoscreening, and immunoblotting reactions
described in the present work.

EXAMPLE 5:

Synthesis and Cloning of Double Stranded cDNA

Poly(A)⁺ kidney mRNA (20 μ g) from Example 3
20 was used as template for cDNA enzymatic synthesis. Avian
myeloblastosis virus (AMV) reverse transcriptase was used
to synthesize the first strand of cDNA in the presence of
oligo-dT₁₂₋₁₈ primer. The conditions described are for
500 μ l reaction mixture with 20 μ g of template. The
25 reaction mixture contains poly(A)⁺ RNA template, 50 mM
Tris, pH 8.3, 10 mM MgCl₂, 100 mM KCl, 140 μ g/ml
primer, 1mM CH₃HgOH to denature RNA, 30 mM beta-mercapto-
ethanol, 1 mM vanadyl sulfate ribonucleoside complex, 2 mM
each of deoxyribonucleotides, and 120 units of reverse
30 transcriptase. Incubation was at 42°C for 1 hour. Free
nucleotides were removed by gel filtration over Sephadex
G100. The RNA template was denatured by treatment with 12
mM CH₃HgOH. The second strand of cDNA was synthesized
using the Klenow fragment of E. coli DNA Polymerase I. The
35 reaction consisted of 100 mM Hepes, pH 6.8, 70mM KCl, 7mM
MgCl₂, 10mM DTT, 22.5mM beta-mercaptoethanol, 0.5mM of

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1 each deoxyribonucleotide, and 100 units of Klenow enzyme
per 2 g of cDNA. The reaction was carried out at 15°C
for 18 hours in a final volume of 1 ml. After phenol
5 extraction, Sephadex G100 gel filtration and ethanol
precipitation, the cDNA was treated with S₁ nuclease to
cleave the hairpin loop at the 5' end of the second strand.
The amount of S₁ required was titrated for each experi-
ment in pilot reactions using alkaline agarose gel electro-
10 phoresis. Optimum concentration was found to be 2.5 units
of S₁ nuclease per ng of double stranded cDNA in this
case. The reaction mixture also contained 30mM NaAc, pH
4.6, 300mM NaCl, and 3mM ZnSO₄ and the incubation was
37°C for 1 hour.

15 The cDNA was then treated with terminal trans-
ferase and dCTP to add 10-15 residues to the 3' end. Pst
I-digested pBR322 was similarly treated with terminal
transferase and dGTP. The reaction mixture contains 140mM
potassium cacodylate, pH 7.2, 0.5mM CoCl₂, 240mM dCTP or
dGTP, 1.5 mg/ml BSA and 6,000 u/ml terminal transferase.
20 The reaction was carried out at 25°C for 15 min. The cDNA
was inserted into the Pst I site of pBR322 by homopolymeric
dC:dG tailing. The oligo (dC)-tailed cDNA and oligo(dG)-
tailed vector were annealed at a molar ratio of 1:2 at
42°C for 2 hours. The recombinant plasmids were trans-
25 formed into E. coli strain C600 by CaCl₂ treatment and
heat shock. Transformants were selected for tetracycline
resistance (Tet^R) and ampicillin sensitivity (Amp^S).

One ml of overnight E. coli C600 culture was
inoculated into 100ml of L broth in a 500 ml flask. The
30 cells were grown at 37°C with vigorous shaking to a density
of about 5x10⁷ cells/ml. The culture was chilled on ice
for 10 min. and then centrifuged at 4,000xg for 10 min at
4°C. The supernatant was discarded and the cells were
suspended in (1/5 of the original culture volume) ice-cold,
35 sterile solution of 100mM CaCl₂ and 20mM sodium acetate,

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1 pH 6.5. The resulting suspension was kept on ice for 20
min, then centrifuged again for 10 min. The supernatant
was discarded and the cells were resuspended in (1/100 of
5 the original culture volume) ice-cold sterile solution
of 0.1M CaCl_2 and 20mM sodium acetate. The suspension
was kept on ice for 20 hours.

To 100 μ l of the cell suspension, 1-10 ng of
vector were added in each of ten tubes. The mixture was
left on ice for 10-30 minutes. 1 ml of LB broth was added
10 to each tube and incubation was carried out at 37°C for one
hour with shaking, to allow the bacteria to recover and to
express antibiotic resistance.

Transformants were selected for tetracycline
resistance (Tet^R) and ampicillin sensitivity (Amp^S) on
15 LB-Tet plates (10g bacto-tryptone, 5g yeast extract, 5g
NaCl, 3.5 ml (1M) NaOH and 15g agar per liter containing 25
 $\mu\text{g/ml}$ tetracycline), and on LB-Amp plates (containing 100
 $\mu\text{g/ml}$ of ampicillin, instead of tetracycline). Colonies
began to appear 12-16 hours after onset of 37°C incubation.
20 The transformation frequency was 5×10^5 transformants
per microgram of cDNA on selection for tetracyclin resis-
tance. Approximately 95% of the transformants were both
tetracycline-resistant and ampicillin-sensitive.

EXAMPLE 6:

25 Enrichment of Ep-mRNA and Synthesis of [^{32}P] cDNA probe
Methylmercury Hydroxide Agarose Gel Electrophoresis

Poly(A)⁺ RNA (50 μg) was fractionated by
electrophoresis in a 1.5% agarose gel containing 12.5 mM
 CH_3HgOH using low melt agarose (BRL, Bethesda, MD), at 40
30 volts for 15 hours. Gel lanes containing size markers
(ϕ X174 Hae III digest and ribosomal RNA's of HeLa cells)
were soaked in 0.5 M ammonium acetate and stained with
ethidium bromide. Gel lanes containing poly (A)⁺ were
soaked in 100 mM DTT to allow renaturation of RNA. The
35 lanes were sliced into 30 fractions and the RNA was ex-
tracted from these fractions by controlled microwave

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1 heating and phenol extraction, followed by ethanol precipi-
tation. Portions of the fractionated RNA's were translated
in vitro and immunoprecipitation of the translation pro-
ducts was carried out as described in Example 4 to locate
5 the fraction(s) enriched in Ep mRNA. The ³⁵S-labeled
translation products and their immunoprecipitates were
analyzed by SDS-PAGE. The majority of Ep mRNA was resolved
in fraction number 11 (Figure 3, lane 11), as detected by
immunoprecipitation of the translation products with
10 Anti-Ep 7A7 (Figure 1, lanes (4) and (5)). Using ribosomal
RNA and ϕ XDNA Hae III fragments as markers, fraction
number 11 corresponds approximately to 1400 bp in size.
This fraction was used to synthesize ³²P-labeled single-
stranded cDNA according to Example 5. A specific radio-
15 activity of 10^7 cpm/ μ g was obtained. The ³²P-labeled
cDNA was used as a probe for the initial screening of
recombinant plasmids.

EXAMPLE 7:

Initial Screening of the cDNA Library:

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In Situ Colony Hybridization

Tet^R, Amp^S transformants from Example 5 were
individually picked and grown on nitrocellulose filters
(Millipore, 4.5 cm containing 100 gridded squares). 100
colonies were inoculated onto each of 12 filters, each
25 colony within a grid square. Each filter was placed on the
surface of an LB-Tet plate and incubated at 37°C for 20
hours. The transformants were screened by a modification
of colony hybridization using ³²P-labeled cDNA synthe-
sized from size-fractionated mRNA enriched in Ep message.

30

Filters bearing colonies were treated with 0.4
N NaOH, neutralized, and treated with proteinase K. The
DNA was fixed to the filter by baking at 80°C in vacuo for
4 hours. Twelve filters were hybridized together in 3 ml of
probe at 1×10^6 cpm/ml in 50% deionized formamide (DF)
35 and 0.75M NaCl/75mM sodium citrate at 37°C for 24 hours.
The filters were washed six times with 0.3M NaCl/30mM

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1 sodium citrate at room temperature (40 min./wash), blotted
dry, and exposed to film.

5 A typical filter bearing the colony hybridization
results is shown in Figure 4A, and two classes of positive
colonies were detected. One, comprising 0.1 to 0.4% of the
total colonies, hybridized very strongly to the probe and
exhibited dense spots on autoradiography. The second
class, which consisted of 5% of the total colonies, hybrid-
10 ized to the probe in varying degrees but significantly less
strongly than did the first class. The remainder of the
colonies were negative. By this preliminary screening,
about 95% of the transformants were eliminated from further
screening.

EXAMPLE 8:

15 Immunological Screening of Colonies
and Characterization of Plasmid DNA

Immunological screening was carried out by in
situ colony radioimmunoassay (RIA) with monoclonal anti-
bodies to human Ep using the general method of Helfman et
20 al., supra. Bacterial colonies were grown on 4.5 cm
nitrocellulose filters as described above, and lysed over
CHCl₃ vapor for 30 minutes. Each filter was treated with
10 ml of lysis buffer in a Petri dish at room temperature
overnight with gentle shaking. The lysis buffer contains
25 3% bovine serum albumin (BSA), 50 µg/ml lysozyme, 2 µg/ml
DNase in 50mM Tris HCl pH 7.4./150 mM NaCl (Tris/saline).
The filter was rinsed thoroughly with Tris/ saline and
incubated at room temperature for one hour with 5 ml
of purified IgG 7A7 (1mg/ml) in Tris/saline/3% BSA. The
30 filters were washed six times with the same buffer at 45
minutes per wash to remove nonspecifically adsorbed anti-
body. Bound antibody was detected by a one hour incubation
with ¹²⁵I-labeled affinity purified goat anti-mouse IgG

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1 (1 x 10⁶ cpm/ml). The filters were washed extensively
(six to eight washes) with buffer W, and analyzed by
autoradiography. Positive clones were verified by direct
colony RIA using ¹²⁵I-labeled monoclonal anti-Ep (2 x
5 10⁶ cpm/ml). Radioiodination was carried out by the
lactose peroxidase method. In all RIA's, a filter spotted
with various amounts of purified Ep was included as a
control for the specificity of the immunological detection
of the antigen. In such controls, <1 ng of purified Ep
10 could be detected.

Positive recombinants from colony hybridization
were picked, and grown on gridded nitrocellulose filters in
a registered fashion for immunological screening by in situ
colony RIA with 7A7. This procedure relies on expression
15 of the cDNA inserted in the pBR322 beta-lactamase operon to
produce a fused polypeptide containing the appropriate
antigenic site for the Anti-Ep recognition. From 1.4 x
10⁵ transformants, three positive clones were identified
that reacted consistently with 7A7. These clones were
20 designated pEp1, 2, and 3. A representative filter showing
the detection of such a positive clone (pEp2) is seen in
Figure 4B. The immuno-specificity of the positive clones
was further confirmed by direct colony RIA using ¹²⁵I-
labeled 7A7. All three clones reacted positively and
consistently with [¹²⁵I]7A7. The plasmid DNA of these
25 clones was isolated and its size determined, as described
below.

Plasmid DNA was prepared by the alkaline lysis
procedure of Birnboim, H.C. et al, A Rapid Alkaline Extrac-
30 tion Procedure for Screening Recombinant Plasmid DNA, Nuc.
Acid Res. 7:1513, 1979, and purified by centrifugation
through 1M NaCl in a SW 50.1 rotor at 40,000 rpm for 6
hours at 20°C. Digestion with restriction endonucleases
was carried out under conditions recommended by the sup-
plier. Gel electrophoresis was carried out in 6% poly-
35 acrylamide gels or in 1% agarose gel. ØX174 RF DNA -

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Hae III digested fragments were used as markers. The size of the inserts are 1,400, 600 and 200 base pairs for pEp1, 2 and 3 respectively (Figure 5, lanes 2, 4 and 1 respectively).

EXAMPLE 9:

Hybridization Selection of mRNA and In Vitro Translation

Selection of mRNA was conducted according to the procedures modified from Parnes et al., supra. Plasmid DNA was suspended in H₂O at 1 mg/ml, denatured by heating 2 minutes at 96°C in 0.25 N NaOH, cooled quickly, neutralized with HCl, and spotted to saturation on nitrocellulose. The filters were baked for 2 hours at 90°C and then hybridized with human kidney poly(A)⁺ RNA at a ratio of 2.5 µg poly(A)⁺ RNA per µg of plasmid DNA. Hybridization was carried out at 50°C for 3 hours in a total volume of 100 µl containing 65% (v/v) DE/20 mM 1,4-piperazine-diethanesulfonic acid (PIPES), pH 6.4/0.2% SDS/ 0.4. M NaCl / 100 µg per ml calf liver tRNA. The hybrid selected mRNA was eluted with 200 µl of H₂O at 100°C for 90 seconds and then snap frozen in liquid nitrogen. The eluate was precipitated with ethanol using 10 to 20 µg of calf liver tRNA as a carrier. The hybrid selected mRNA was translated in vitro and the ³⁵S-labeled translation products were resolved by SDS-PAGE and analyzed by fluorography (Figure 6). As seen in Figure 6A the RNAs selected by pEp1, 2 and 3, all directed the synthesis of four ³⁵S-labeled polypeptides (lanes 4, 5 and 6 respectively). The molecular weight of these polypeptides relative to the gel markers were approximately 92,000, 66,000, 29,000 and 15,000 daltons. The 92,000 dalton band was also seen in the endogenous translation (lane 0) and in the pBR322 selected sample (lane 1) but at a much lesser intensity. The 66,000, 29,000, and 15,000 dalton polypeptides were not detected in lanes (0) and (1), indicating that they are Ep-specific.

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1 Final confirmation of the identity of these polypeptides was made by immunoblotting and competitive immunoprecipitation as described in Example 10.

EXAMPLE 10:

5 Detection of Antigenic Proteins Synthesized in vitro by Immunoblotting, and Competitive Immunoprecipitation

Electrophoretic transfer of proteins was carried out according to the general procedure of Towbin et al., supra. Nitrocellulose, 0.45 μ m pore size was used. The transfer was conducted in 25mM Tris HCl pH 8.4/192 mM glycine /20% methanol (v/v) at 0.35 amperes for 12 hours and then at 1 ampere for 3 hours using a Hoefer TE42 Transphor unit with a TE 50 power supply. The electrophoretic blot was rinsed with Tris/saline and incubated with 3% BSA/Tris/saline for 1 hour at 40°C to saturate the remaining protein binding sites. It was then incubated with 7A7 (1 mg/ml) in 3% BSA/Tris/saline for 1 hour at room temperature or overnight at 4°C. After washing with Tris/saline, the blot was incubated with 125 I-labeled goat anti-mouse IgG (1.2×10^6 cpm/ml) for 1 hour at room temperature. For every 100 cm² blot, 10 ml of the antibody solution was used. The blot was thoroughly washed with buffer W six to ten times at 45 minutes per wash and exposed to film. The results are seen in Figure 6B. Two polypeptides, M.W. 29,000 and 15,000 were immuno-blotted by the Anti-Ep whereas the M.W. 92,000 and 66,000 polypeptides were not detected. This is true in the translation of total kidney poly(A)⁺ RNA (lanes 2 and 3) as well as in the hybrid selected mRNA (lanes 4, 5 and 6). Whether this is due to inefficient transfer of high molecular weight proteins or a lack of antigenic recognition remains to be studied.

Competitive immunoprecipitation of 35 S-labeled, hybrid selected translation products was carried out by the addition of purified unlabeled native Ep to the immune reaction mixture. Both the 35 S-labeled M.W. 29,000 and

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1 15,000 polypeptides were immunoprecipitated by 7A7 in the
absence of unlabeled Ep (Figure 7, lane 3). Precipitation
of these polypeptides was inhibited by the addition of
excess unlabeled Ep (lanes 4 and 5). Preimmune mouse serum
5 did not precipitate these polypeptides (lane 1) nor was any
precipitation detected in endogeneous translation (lane 2).
About 50% inhibition resulted in the presence of 2 μ g of
unlabeled purified Ep (lane 4) and over 90% of inhibition
was observed upon the addition of 10 μ g of purified
10 unlabeled Ep (lane 5). These results indicate that of the
hybrid selected translation products, the 29,000 and
15,000 dalton polypeptides are recognized by monoclonal
Anti-Ep, 7A7 and that authentic Ep competes with them for
antibody binding.

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APPENDIX AEp Purification

According to the present method, unconcentrated or concentrated urine from severely anemic patients can be used as the raw material. Starting samples, are preferably first centrifuged to eliminate insoluble material and purified preferably by hydrophobic interaction chromatography (HIC), as described by Lee-Huang, S.: A New Preparative Method for the Isolation of Human Erythropoietin With Hydrophobic Interaction Chromatography, Blood 56:620-624, 1980, in order to remove the bulk of urinary contaminants and permit more efficient and repeated use of the immuno-adsorbents. HIC involves processing of the raw material through a crosslinked neutral gel chromatographic column wherein the gel contains a hydrophobic group. Phenyl-Sepharose CL4B is particularly preferred because it provides a strong yet easily reversible binding with Ep. Octyl-Sepharose may also be used, but Ep elution therefrom is less complete. The specific activity of Ep obtained from this step depends on the potency of the starting material but generally ranges between about 115 and 250 units per mg of protein. The yield is usually about 80%. One unit of Ep is defined as the activity contained in 0.5 mg of the second International Reference Preparation of Human Urinary Erythropoietin (IRP) (obtained from the World Health Organization, International Laboratories of Biological Standards, Hampstead, London, England), or one-tenth of the contents of one ampule of this preparation.

The HIC-purified material can be used as the immunogen to raise antibodies to Ep (hereinafter designated as "Anti-Ep") and its common contaminating impurities (hereinafter designated as "Anti-I"). This can be conveniently performed in a single immunization using antibody-producing laboratory animals. The immunization is carried out in accordance with methods well known in the art and, in the case of Ep or other weak immunogens, it preferably includes several booster injections in addition to the

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1 initial injection. Anti-Ep titers are determined by the in
vivo exhypoxic polycythemic mouse bioassay described by
Camiscoli, J.F. and Gordon, A.S.: Bioassay and Standardi-
5 zation of Erythropoietin in Gordon, A.S. (Ed.) Regulation
of Hematopoiesis, Meredith Corp., New York, 1970 pp 370-396.

Polycythemia is induced in mice by hypobaric
hypoxia. In order to keep a high protein concentration and
thus stabilize the Ep activity, Ep samples for assay are
made up in a buffered albumin solution. Samples are in-
10 jected into mice posthypoxia, intraperitoneally. Ep
activity is measured by its stimulation of ^{59}Fe incorpo-
ration in red blood cells. ^{59}Fe incorporation is
determined in a gamma counter. The results are compared to
those obtained using the second IRP from WHO. Anti-Ep
15 titers are determined by assaying for ability to neutralize
Ep-stimulated ^{59}Fe incorporation in red blood cells.

The immunized laboratory animals are then finally
bled. Antisera from the bleedings after the last injection
are isolated, assayed for anti-Ep titers, and purified by
20 immunoaffinity chromatography to eliminate non-immunoglobu-
lins. The rabbit antisera are processed through a Sepharose
4B column to which goat-(anti-rabbit) Igs have been cova-
lently linked. The non-immunoglobulins are excluded from
the column, while the specific Igs are eluted with, e.g.,
25 3M sodium thiocyanate (NaSCN) or 0.2M acetic acid.

The thus obtained specific immunoglobulin prepa-
ration is treated to separate Anti-Ep from Anti-I. For
this purpose, a highly purified Ep preparation is preferably
used. However, the present invention does not require pure
30 Ep for antibody preparation and/or separation. Partially
purified Ep (or other partially purified antigen), prepared
according to conventional methods, is adequate for carrying
out the method of the present invention.

The antibody separation may be preferably accomp-
35 lished by a new principle and procedure which employs
reversible binding of antigen to a supporting matrix and

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1 thus permits subsequent recovery of valuable Ep (or other antigen) after it is used in the Anti-Ep (or other antibody) purification, without substantial loss of activity.

5 The antibody separation procedure utilizes the fact that Wheat germ Lectin-Sepharose 4B (WGLS) columns coated with purified Ep have differential affinity for their biospecific and immunospecific ligands. The procedure involves four steps:

10 1. Purified Ep is bound to WGLS to produce a WGLS-Ep complex. Ep binds tightly to WGLS due to interaction of its N-acetyl-glucosaminyl residues with the wheat germ lectin.

15 2. The affinity purified rabbit immunoglobulins are processed through a WGLS-Ep column: Anti-Ep binds to the WGLS-Ep complex, while Anti-I does not, but is excluded in the effluent and set aside for further use. Of course, since the original purified Ep, that was used to coat the WGLS column in Step 1, was not homogeneous, its impurities will also be carried over to the WGLS column of Step 1 and, consequently a small fraction of the Anti-I will bind to the WGLS-Ep column of Step 1. This was the shortcoming of conventional immunoaffinity techniques which the present invention has overcome, as will be described below.

20 Anti-Ep bound to the WGLS-Ep complex is eluted and preferably processed again through a regenerated WGLS-Ep column to insure complete resolution of Anti-Ep/Anti-I immunoglobulins. The Anti-I-containing eluents from the first and the second separation are pooled and Anti-I are recovered therefrom.

30 3. Since the affinity between the constituents of the immune complex (Ep-(Anti-Ep)) is lower than the affinity between Ep and the sugar-lectin complex (WGLS-Ep), Anti-Ep from WGLS-Ep-(Anti-Ep) can be selectively eluted using a weak acid or a dissociation reagent. The ability of WGLS to bind Ep both at low pH and under dissociating conditions makes WGLS a useful adsorbent for Anti-Ep

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1 purification and at the same time enables recovery of the
valuable Ep (see step 4 below). The thus recovered Anti-
Ep is separated from the eluent (e.g., by dialysis) lyophi-
lized, and stored frozen for subsequent use.

5 4. Ep can be recovered from WGLS-Ep, once Anti-
Ep has been eluted, by further elution, preferably with
N-acetylglucosamine or N,N-diacetylchitobiose. This is not
possible under conventional immunoaffinity procedures
since, normally, the immunoabsorbent is irreversibly
10 coupled to the supporting matrix and cannot be recovered.
When the supply of the antigen (used as the immunoabsorbent)
is limited, the recovery of such materials is a very
valuable saving. Alternatively, the column of WGLS-Ep can
be regenerated and can be reused.

15 The thus recovered Anti-Ep and Anti-I are sepa-
rately covalently linked to CNBr-activated Sepharose 4B.
The coupling procedure has been generally described by
Axen, R. et al "Chemical Coupling of Peptides and Proteins
to Polysaccharides by Means of Cyanogen Halides" Nature,
20 214:1302-1304, 1967. The Sepharose-(Anti-Ep) and Sepharose-
(Anti I) so prepared are used in column form for the direct
immunoaffinity chromatography (DIAC) and reversed immunoaf-
finity chromatography (RIAC) purification of Ep.

25 The Ep purified by HIC is further purified by
DIAC on a Sepharose-(Anti-Ep) column. This purification
results in exclusion of the majority of contaminants from
the column, which are carried off in the effluent, while Ep
is retained on the column. It is important to note, how-
ever, that at this stage some antibodies to some minor
30 impurities will be present in the Sepharose-(Anti-Ep)
column because of the lack of homogeneous Ep in the immuno-
affinity purification of the Anti-Ep. This is the intrinsic
limitation of any conventional direct immunoaffinity
technique.

35 Ep from the Sepharose-(Anti-Ep) column is eluted
with an appropriate buffer. Choice of buffer is important

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1 in preserving Ep activity. For example, commonly used
immune complex dissociating acidic buffers or chaotropic
ions (such as glycine hydrochloride buffer or sodium thio-
cyanate) inactivate Ep, while simple alkali gives incomplete
5 desorption. The present inventor has found that inclusion
of 10-20% of a polarity reducing agent (such as glycerol or
another common 1,2-glycol) and a dissociation agent (such
as guanidine hydrochloride or urea) in an alkaline eluant
(such as NaOH) facilitates effective release of Ep from the
10 immunoadsorbent while preserving Ep activity. Preferred
are ethylene glycol and guanidine hydrochloride, which can
be easily removed and which appear to have no detrimental
effect on Ep activity.

The thus eluted Ep is dialyzed (preferably immedi-
15 ately and thoroughly) against water and sodium phosphate
buffer. Under these conditions, DIAC is very efficient,
offering a high purification factor (usually about 169-fold
over HIC) and a high yield (usually about 80% or higher).
However, the main limitation of DIAC is the impurities in
20 the original Ep preparation. The antibodies against these
impurities are carried over in the purification system and
immunoadsorb their antigens in the Sepharose-(Anti-Ep)
column. As a consequence, the purity of the DIAC product
cannot exceed that of the original Ep used in preparation
25 of the WGLS column (Step 1) for antibody purification.

At this point further purification is accomplished
with another Sepharose column coupled with Anti-I. While
Anti-Ep contains only a minor fraction of antibodies to the
impurities, Anti-I consists of the bulk of these antibodies.
30 Thus, the Sepharose-(Anti-I) column will be able to provide
sufficient antibody sites to bind substantially all the
impurities contained in the DIAC-purified Ep.

Upon loading DIAC-purified Ep onto a Sepharose-
(Anti-I) column, the trace impurities are retained in the
35 column due to the formation of specific immune complexes
with their corresponding antibodies, which are present in

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1 great excess on the column, whereas pure Ep is selectively
excluded in the effluent. This step affords preparation of
Ep which is purier than the original antigen. Such effi-
5 ciency is not attainable with other conventional immunoaf-
finity techniques. This immunoaffinity chromatography
step wherein the impurities are bound to their antibodies,
while the valuable protein is excluded in the effluent, is
referred to as Reverse Immunoaffinity Chromatography (RIAC).

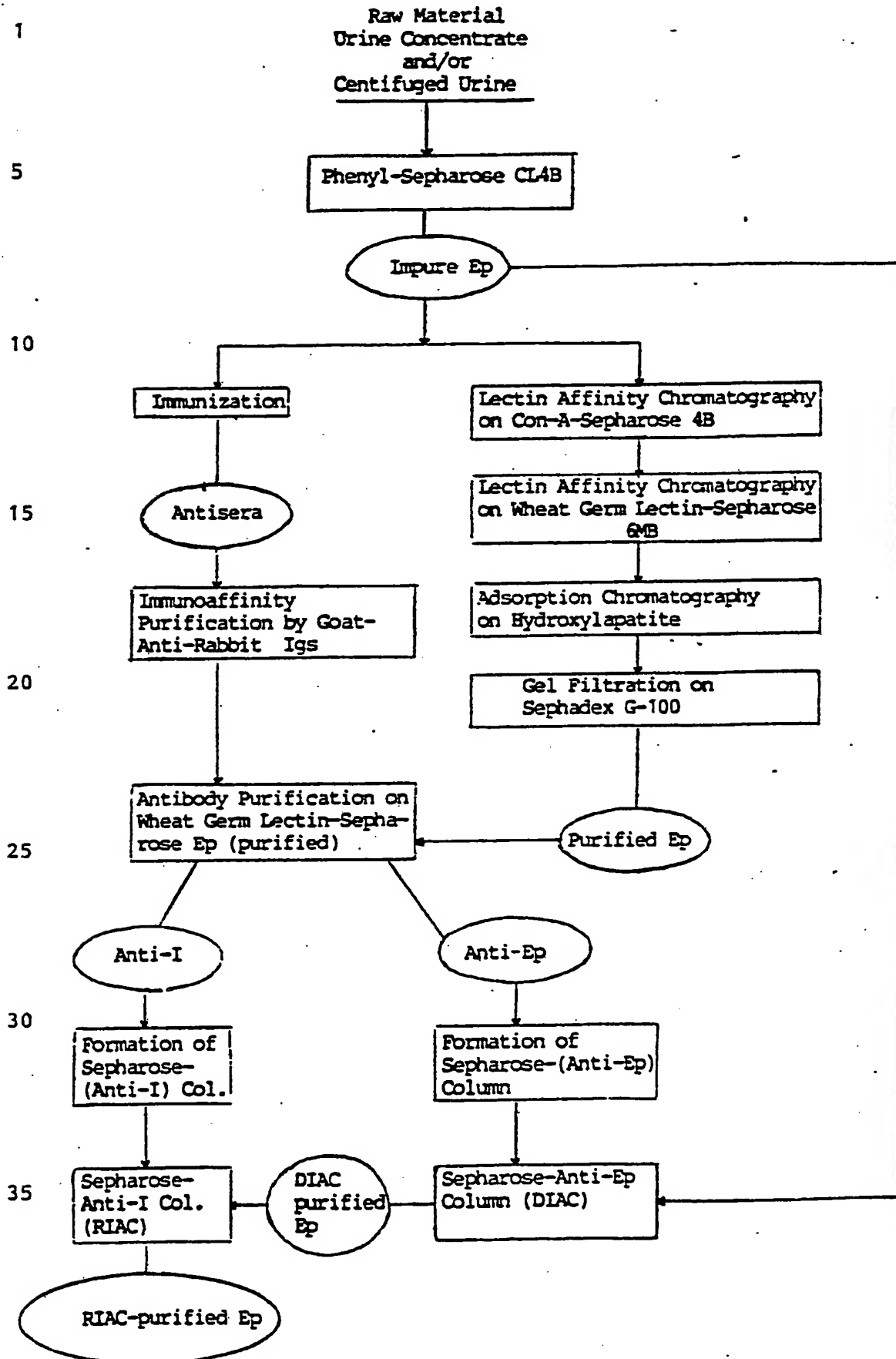
The impurities removed in the reverse immunoaf-
10 finity step are a constant set of residual urinary contami-
nants; and they have been copurified with Ep in many
separation techniques, and are therefore fairly uniform
from batch to batch. Thus, crude urine from a source
different from that employed to generate the antisera can
15 be effectively purified by the HIC-DIAC-RIAC procedure.
The amount of Anti-I required for immunoadsorption of these
minor impurities of DIAC-purified Ep is small relative to
the total capacity of the Sepharose-(Anti I) column.
Furthermore, since reverse immunoaffinity chromatography
20 immunoadsorbs only the contaminating impurities, no desorp-
tion of Ep is required, thus minimizing manipulation of
valuable samples and increasing yield accordingly. The
impurities retained on the column can be subsequently
dissociated from the immunoadsorbent by eluting with an
25 appropriate acidic eluent. The column is thus regenerated
and ready for subsequent use.

A flow chart outlining the various steps of the
present invention is set forth on the following page.

30

35

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1 The DIAC-RIAC purified Ep can be tested for
homogeneity by attempting further purification using con-
ventional purification techniques (preferably chromato-
graphic techniques and/or gel filtration), and assayed for
5 biological activity.

 The DIAC-RIAC purified Ep is further tested for
homogeneity and characterized by electrophoretic techniques,
such as gel electrophoresis, isoelectric focusing, and disc
electrophoresis in non-dissociating systems according to
10 well-known methods described by: (a) Laemmli, U.K.: Cleavage
of Structural Proteins During the Assembly of the Head of
Bacteriophage T₄, Nature 227: 680-685, 1970; (b) Catsim-
poolas, N. et al (Ed.); Biological and Biomedical Applica-
tion of Isoelectric Focusing, New York, Plenum Press, 1977,
15 and (c) Davis, B.J.: Disc Electrophoresis-II: Method and
Application to Human Serum Protein, Ann. N.Y. Acad. Sci.
121:404-427, 1964.

 The following examples serve further to illustrate
the present invention, but not to limit its scope.

20 Materials: Phenyl-Sepharose CL4B, ConA-Sepharose
4B, Wheat germ Lectin-Sepharose 6MB, CNBr Activated Sepha-
rose 4B Sephadex G100 were obtained from Pharmacia Labora-
tories, Inc., (Piscataway, New Jersey). Guanidine hydro-
chloride (ultra-pure) was obtained from Schwartz-Mann
25 Biochemicals (Spring Valley, New York). Ethylene glycol
and N-acetylglucosamine were from Sigma Chemical Company
(St. Louis, Missouri). All other chemicals were from
Fisher Scientific Company (Fairlawn, New Jersey), except
when otherwise specifically indicated.

30 Concentration of all column eluates was carried
out at 4°C using an Amicon ultrafiltration apparatus with
YM10 membrane unless otherwise specified.

35

APPENDIX BMonoclonal Anti-Ep

Ep is a weak immunogen. Accordingly, availability of pure Ep and choice of the initial immunization procedure (animals, sites and schedules) may substantially affect the efficiency of hybridoma production and the ability of the hybridoma to reliably secrete monoclonal Anti-Ep having the requisite properties.

Human Ep isolated from urine of anemic patients by Hydrophobic Interaction Chromatography on Phenyl-Sepharose CL4B and subsequently purified by Direct Immunoaffinity Chromatography followed by Reverse Immunoaffinity Chromatography (as described in the Ep Purification Patent Application) is used as the immunogen. The purity of the thus purified Ep was compared to that of homogeneous Ep provided by another investigator. The results are shown in Figure 1. The material used in this work as the antigen also shows a single band by gel isoelectric focusing and by electrophoresis under nondissociating conditions as described fully in the Ep Purification Patent Application.

In vitro immunization and subsequent fusion resulted in unstable hybrids secreting IgM. The combination of in vivo primary injection and in vitro boosting also resulted in unstable hybrids.

In vivo immunization is, therefore, preferred. Female laboratory mice are preferably used. Multiple injections in multiple sites increase the probability of obtaining a satisfactory immune response. Immune response is assayed, preferably, by Solid Phase Radioimmunoassay (SPRIA) according to the procedure of Klinman, N.R. "The Mechanism of Antigenic Stimulation of Primary and Secondary Clonal Precursor Cells" J. Exp. Med. 135:241-260 (1972), as set forth in detail in Example 2, below.

The Ep-neutralizing ability of immune mouse serum is preferably assayed by the in vivo exhypoxic polycythemic

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1 mouse method, as described in the Ep Purification Patent
Application, as said method is the most reliable (though
also the most expensive and time-consuming). The mice with
an acceptable immune response are selected. Immune re-
5 sponse is measured by Anti-Ep titer and Ep-neutralizing
titer. For the response to be considered acceptable,
Anti-Ep titer should show at least 50% binding at 1:10,000
serial dilution (Figure 1A) and Ep-neutralizing titer
should show neutralization of over 100 Ep units/ml of mouse
10 serum. These performance characteristics are rather
formidable, considering the weak immunogenic properties of
Ep. Accordingly, the Ep used for immunization should be
the purest possible and the number of mice immunized should
be relatively large. Generally, assuming careful selection
15 and execution of the immunization protocol, about one mouse
in six immunized will exhibit an acceptable immune response.

Hybridomas are fused from spleen lymphocytes of
the mice showing acceptable immune response and from non-
secreting mouse myeloma cells. NS-1, a non-immunoglobulin
20 secreting myeloma cell line of Balb/c origin, commercially
available (from Mutant Cell Repository, Institute of Medical
Research, Camden, N.J.) and resistant to 8-azaguanine is
preferred. Other types of myeloma cell lines that, in
principle, would also be suitable include those discussed
25 in Kohler, G., Howe, S.C., and Milstein, K.; Fusion
Between Immunoglobulin-Secreting and Non-Secreting Myeloma
Cell Lines, Eur. J. Immunol. 1976, 6:292-295.

Fusion is carried out according to the general pro-
cedure of Kennett, R.H. in Monoclonal Antibodies, Hybri-
30 domas: A New Dimension in Biol. Analyses (Kennett et
al, Eds.) Plenum Press, 365-367 (1980), with such modifica-
tions as are set forth in Example 2, below.

Fusion hybrids are fed and grown in selective
media. Surviving hybridoma cells from these media are
35 propagated in culture and their culture media are screened
for the presence of Anti-Ep by solid phase radioimmunoassay

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1 (SPRIA) in accordance with the method of Klinman, supra.
Positive hybridoma cultures are then assayed for Ep-neutralization, preferably by the exhypoxic polycythemic mouse method.

5 After Ep-binding and/or Ep-neutralization have been confirmed, the positive hybridoma cultures are immediately cloned and grown in liquid media. Immediate cloning and recloning are necessary to ensure hybridoma stability. It is customary in the art not to consider a
10 hybridoma culture stable until 100% of clone colonies derived from positive cultures upon recloning are also positive. The stability of hybridoma cells of the present invention has been confirmed by prolonged storage (1 to 2 years) without decline in Anti-Ep secretion. Three
15 stable clones were thus isolated (hereinafter designated as 7A7, 7B9, and 2A10) from 6460 hybridomas in a total of 10 fusions.

After successful cloning of Anti-Ep-secreting hybridomas, large quantities of high-titered antibodies can
20 be obtained by growing hybridomas in culture, or, preferably, by ascites induction. Ascites tumor can be induced by intraperitoneal injection of cloned hybridoma cells into pristane primed syngeneic mice (about 10^7 hybridoma cells per primed mouse). After ascites has been allowed to
25 develop for several (preferably 2-3) weeks, ascitic fluid is harvested from the peritoneal cavity (multiple collections are possible and desirable, since the objective is to obtain as much hybridoma as possible). Monoclonal Anti-Ep activity of ascitic fluid is tested by SPRIA.

30 In order to confirm that the ascites Anti-Ep activity is in fact due to immunoglobulin, and in order to prepare stable antibody preparations, free of degradative enzymes such as proteases and nucleases, suitable for Ep-purification, for Ep mRNA identification and screening
35 of an Ep-cDNA library, the ascites immunoglobulin must be purified. Ascites immunoglobulin and its subclass are

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1 characterized to help design and develop the most effective purification procedures.

The types of immunoglobulin chains are characterized, preferably by use of an isotype-specific rabbit
5 Anti-mouse immunoglobulin kit (Boehringer-Mannheim, Indianapolis, Indiana). This test is also used to confirm the results after immunoglobulin purification.

The types of the three antibodies corresponding to the three stable clones produced in this work were:
10 IgG2a/k (from 7A7), and IgG1/k (from 7B9 and 2A10).

The antibody purification technique was subject to optimization, as many of the available methods presented serious drawbacks.

Protein A-Sepharose affinity chromatography is
15 generally satisfactory for the purification of IgG2a/k. However, this affinity adsorbent fails to bind IgG1 efficiently (even at a slightly basic pH such as 8.4). Ammonium sulfate fractionation, followed by diethylaminoethyl cellulose (DEAE-cellulose) ionic exchange and Sephadex
20 G-200 gel filtration, fails to remove all contaminating proteases and nucleases. These degradative enzymes are deleterious to the antibodies, to Ep activity (during immunoaffinity chromatography purification using monoclonal Anti-Ep as the adsorbent) and to Ep-mRNA stability (during
25 purification by polysome immunoprecipitation). DEAE-Affi gel blue chromatography does remove protease and RNase effectively but is difficult to optimize (maximum resolution conditions have to be determined separately for each antibody) and often results in low yields. Protein A-Sepharose
30 CL4B affinity chromatography followed by precipitation of the eluted IgG2a with ammonium sulfate, is used for purification of monoclonal Anti-Ep 7A7. The yield is usually at least 90%. Affinity purified goat anti-mouse immunoglobulin covalently coupled to Sepharose 4B is effective for IgG1
35 purification and remove protease and RNase. It can thus be used for the purification of monoclonal Anti-Ep 7B9 and

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1 Anti-Ep 2A10 (IgG1/k). Both monoclonal preparations can
thus be free of detectable degradative enzyme activity.
The thus recovered purified immunoglobulins can be quanti-
tated by SPRIA (by measuring their native Ep-binding
5 ability) and their heavy and light chains can be charac-
terized by SDS-PAGE. Ep-neutralization ability can be
tested in vivo, by exhypoxic polycythemic mouse bioassay.
Preferably, native Ep binding is determined by immunoblot
and ^{125}I -Ep (denatured) binding is determined by immuno-
10 precipitation.

Hybridoma 7A7 is a much stronger Anti-Ep producer
than either 7B9 or 2A10, secreting more than ten times as
much antibody. Of the three antibodies isolated in this
work, only 2A10 neutralizes Ep. All three antibodies
15 recognize (bind to) both native and ^{125}I -labeled Ep.

The difference in the binding affinity between
the monoclonal antibodies of the present invention and the
antibody reported by Weiss, et al., supra, is illustrated
by the fact that, in competitive radio-immunoprecipitation
20 of ^{125}I -Ep with unlabeled Ep, a ratio of native to
labeled Ep of 41 was sufficient to cause 50% inhibition of
 ^{125}I -Ep binding, (as opposed to a ratio of 2500 reported
in Weiss et al). The increased binding affinity of the an-
tibodies of the present invention is extremely significant
because it permits the non-neutralizing antibodies disclosed
25 herein to be used in Ep purification by immunoaffinity
chromatography using purified monoclonal Anti-Ep as the
immunoabsorbent. Immunoglobulin from 7A7(IgG2a) is pre-
ferred because it is secreted in larger quantities, it can
be effectively purified (with a high yield), and it does
30 not neutralize (and thus possibly inactivate) Ep.

Purification of Ep by immunoaffinity chromatog-
raphy (using monoclonal Anti-Ep from 7A7 as the immunoad-
sorbent) yields, in one step, Ep of purity and yield
comparable to those obtained with a combination of DIAC and
35 RIAC. Furthermore, since these monoclonal antibodies also

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1 recognize Ep from other mammals such as sheep, rabbit, rat
and mouse, they would also be very useful in affinity
purification of Ep from those and other species.

In addition to providing improved means for Ep
5: purification, the production and purification of monoclonal
Anti-Ep according to the present invention is expected to
facilitate studies on erythropoiesis. The monoclonal
Anti-Ep of the present invention provides useful probes for
detection of Ep-synthesizing cells, Ep-responsive cells and
10 Ep specific receptors, and can be used advantageously in
conjunction with immunofluorescence and radioimmunoassay
techniques. The regulation of Ep-gene expression under
both normal and pathological states can be studied by the
identification of Ep-mRNA and its translation product using
15 immunoblotting, immunoprecipitation and immunoautoradio-
graphy. Moreover, the monoclonal antibodies of the present
invention are useful probes in the cloning of Ep-gene and
in the identification of the cloned Ep-gene product. Many
of the above uses of monoclonal Anti-Ep have been confirmed
20 in practice, as more fully set forth in the Ep Clone Patent
Application.

The present invention is further described in the
following Examples which are intended to illustrate it but
not to limit its scope.

25 Materials and Sources:

Fetal Calf Serum (FCS) and tissue culture media were
from Gibco, Grand Island, N.Y.

Sodium azide was from Sigma, St. Louis, Mo.

Bovine serum albumin (BSA) was from Schwartz Mann,
30 Spring Valley, N.Y.; and

⁵⁹FeCl₃ was from New England Nuclear, Boston, Mass.

The Antigen: Human Ep was isolated from urine of anemic
patients by Hydrophobic Interaction Chromatography on
Phenyl-Sepharose CL4B and subsequently purified by Direct
35 and Reverse Immunoaffinity Chromatography (DIAC-RIAC).

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What is claimed is:

1. A peptide immunochemically reactive with a monoclonal antibody to human erythropoietin.
2. A peptide according to claim 1, produced by an organism, containing at least one DNA fragment comprising a DNA sequence coding for said peptide.
3. A peptide according to claim 2, wherein said organism has been transformed by an expression vector comprising said DNA fragment.
4. A fusion protein produced by an organism, said protein consisting essentially of a peptide immunochemically reactive with a monoclonal antibody to human erythropoietin and a portion of an endogenous protein of the organism.
5. A DNA fragment having a deoxynucleotide sequence encoding a peptide immunochemically reactive with a monoclonal antibody to human erythropoietin.
6. A recombinant DNA molecule comprising a DNA fragment according to claim 5.
7. An organism transformed by a recombinant DNA molecule, according to claim 6.
8. An organism according to claim 7 comprising a bacterium of the species Escherichia coli.
9. A recombinant DNA molecule according to claim 6 containing said DNA fragment at a site within said molecule suitable for expression of the peptide coded for by said DNA fragment.

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10. A recombinant DNA molecule according to claim 9 wherein said peptide is capable of expression as a fusion protein.

11. A recombinant molecule according to claim 6 wherein said recombinant DNA molecule is a hybrid plasmid derived from pBR322.

12. A recombinant molecule according to claim 11 wherein said DNA fragment is inserted in the Providencia stuartii I cleavage site of said plasmid.

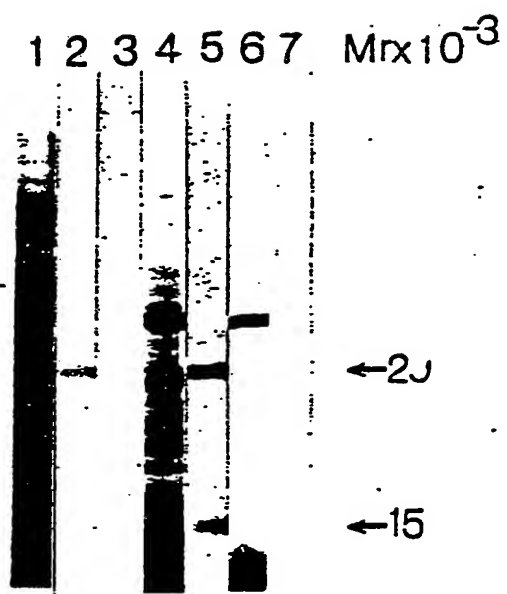
13. A recombinant DNA molecule according to claim 12, wherein said DNA fragment is inserted by homopolymeric dC:dG tailing.

14. A functional mRNA molecule carrying human erythropoietin message, in purified form.

15. A functional mRNA molecule according to claim 14, derived from a renal carcinoma cell, said renal carcinoma having a high erythropoietin titer.

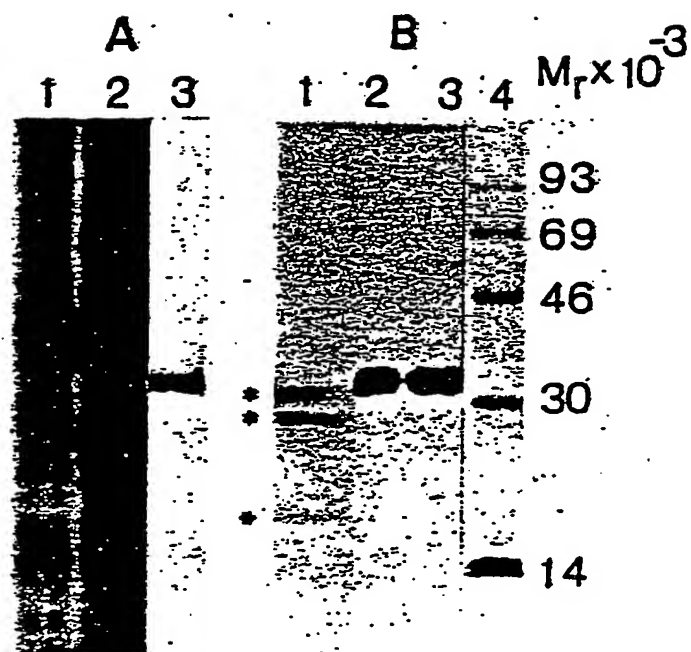
16. A functional mRNA molecule according to claim 15, wherein said titer is greater than about 1 unit/g of tissue.

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FIG. 1

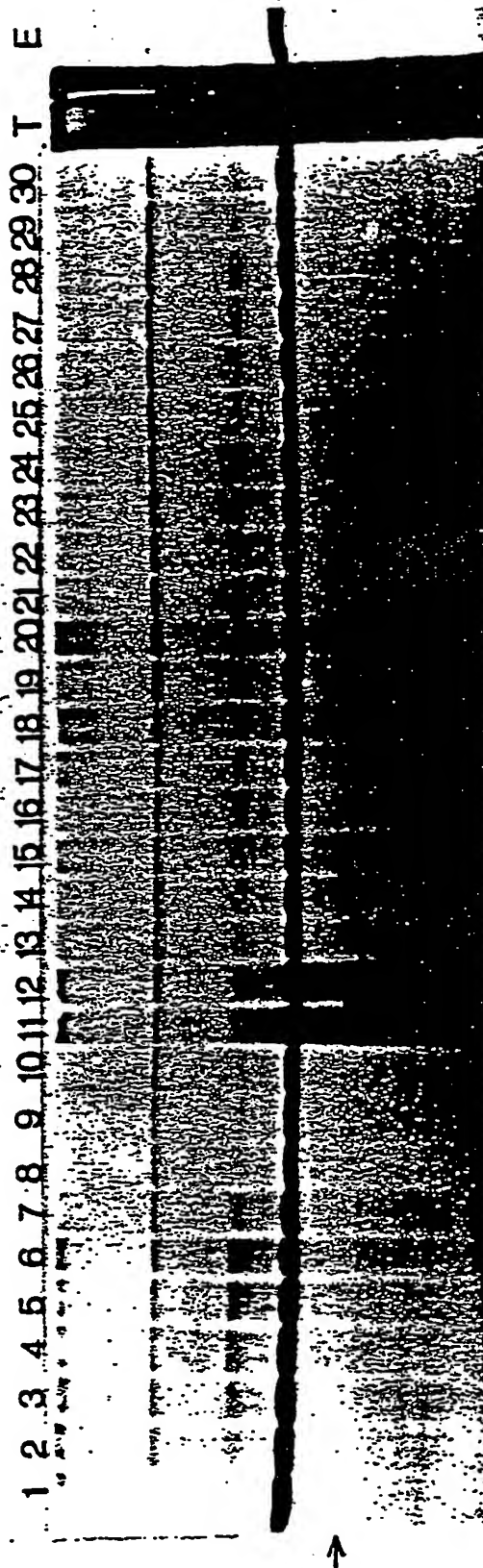
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FIG. 2



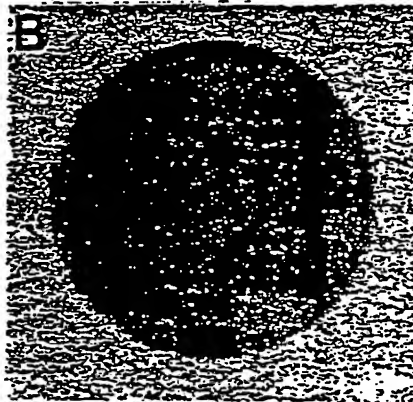
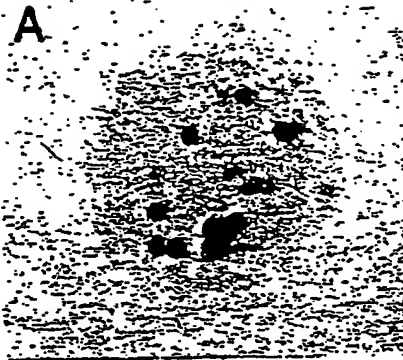
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FIG. 3

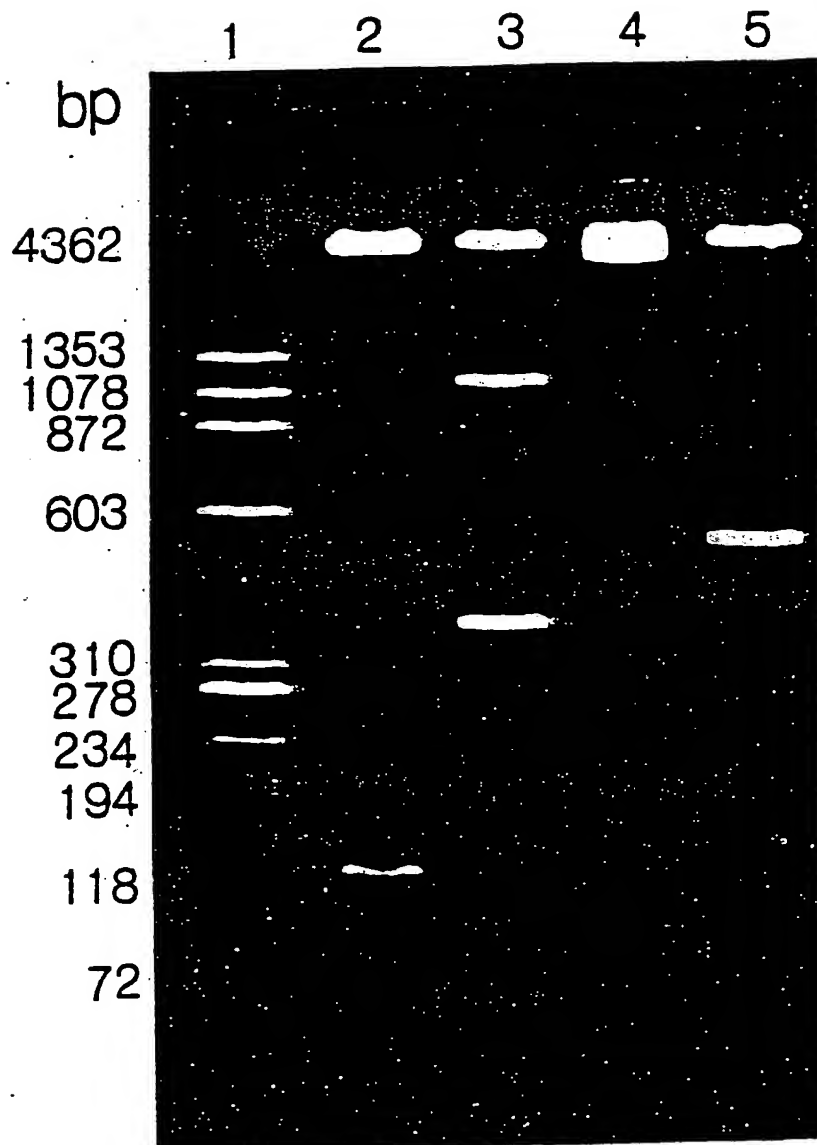


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FIG. 4



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FIG. 5

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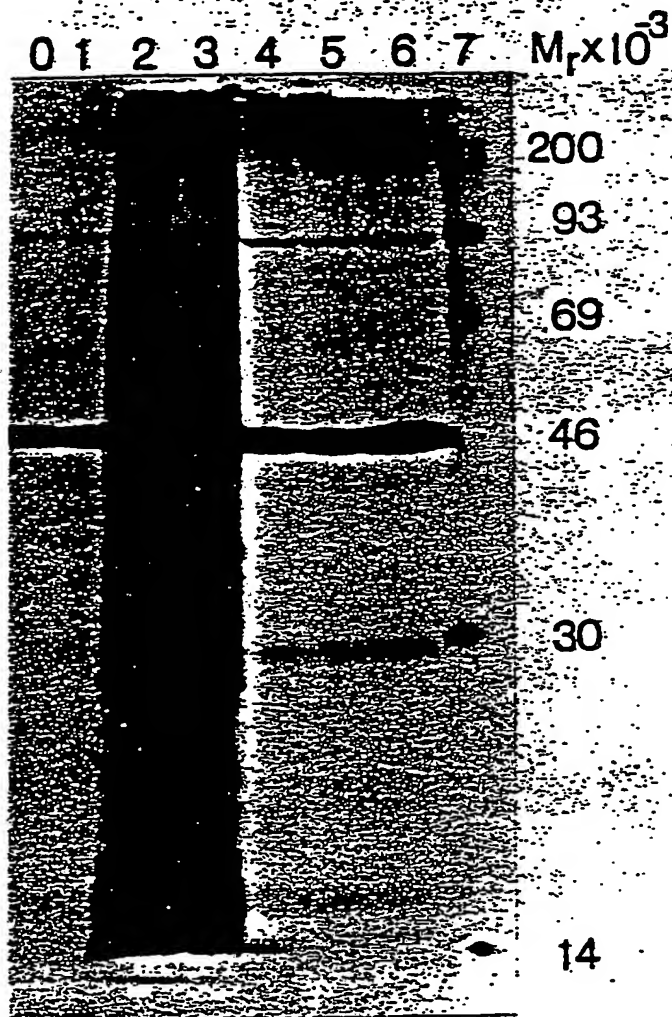
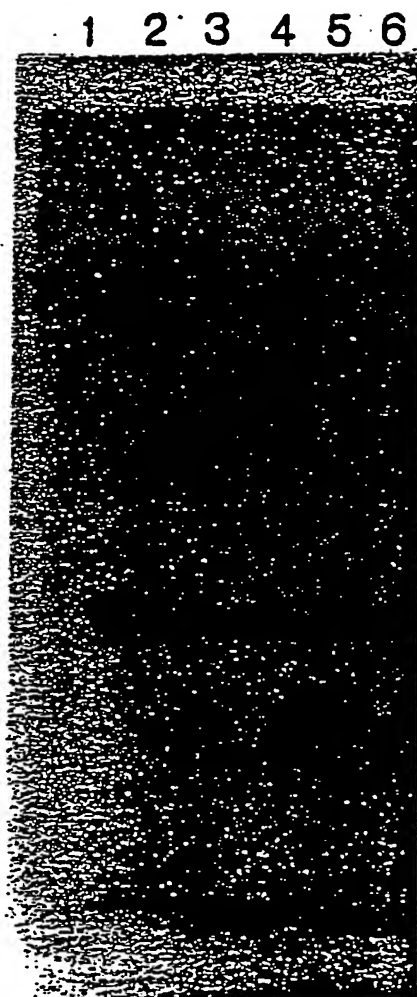
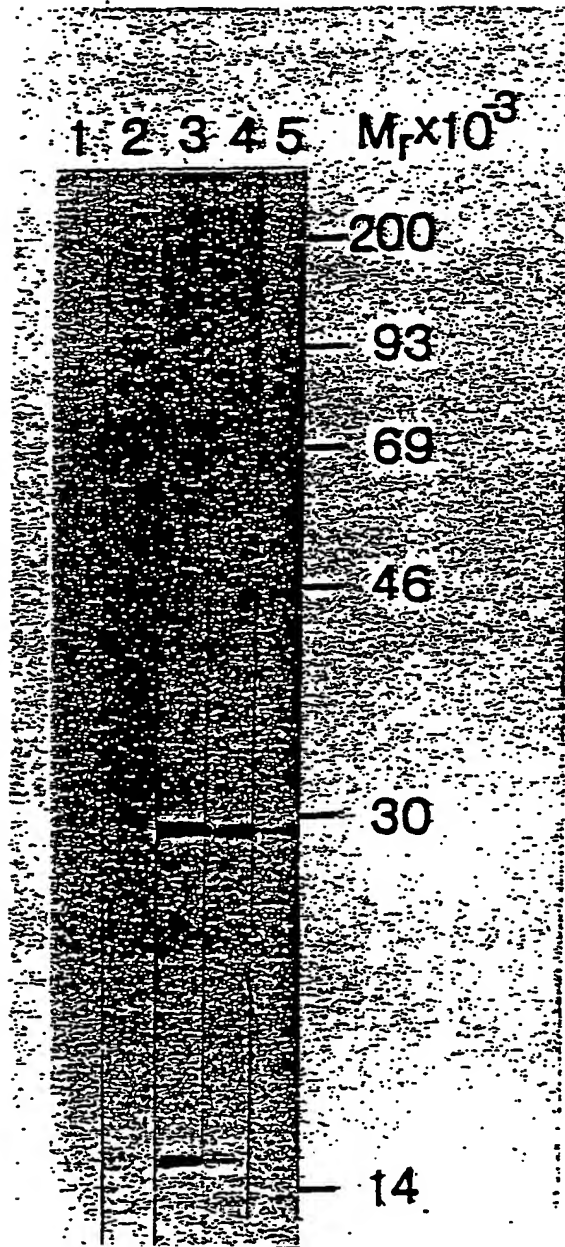
FIG. 6A**FIG. 6B**

FIG. 7



INTERNATIONAL SEARCH REPORT

International Application No PCT/US85/00054

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ³		
According to International Patent Classification (IPC) or to both National Classification and IPC ⁴ 260/112, 536/27, 435/172.3 C07F 15/12 C12N 15/00		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁵		
Classification System	Classification Symbols	
435	68, 172.3, 317	
536	27	
260	112	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁶		
Computer Search-Biosis; Chemical Abstract Index		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category ⁷	Citation of Document, ¹⁵ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
T	Lee-Huang 1984 "Cloning and Expression of Human Erythropoietin cDNA in <i>E. coli</i> " Proc. Natl. Acad. Sci. V 81 p2708-12	1-16
Y	Lee-Huang 1982 (Abstract) "Monoclonal Antibodies to Human Erythropoietin" Fe. Proc. V 41 p520	1-16
X	Lin et al 1984. (Abstract) "Cloning of the Monkey Erythropoietin Gene" J. Cell. Biochem (Supp 8B) p45	5-16
Y	UK Application 2,085,887 6 May 1982 Hayashibara et al "Process for the Production of Human Erythropoietin"	1-4
Y	US 4,377,513 22 Mar 1983 Sugimoto et al. "Process for the Production of Human Erythropoietin"	1-4
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁶ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"A" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ¹⁹ May 6, 1985	Date of Mailing of this International Search Report ² 07 JUN 1985	
International Searching Authority ¹ ISA/US	Signature of Authorized Officer ²⁰ 	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y

US 4,503,151 Paddock et al 5 Mar. 1985
 "Recombinant cDNA Construction Method and
 Hybrid Nucleotides useful in Cloning"

5-16

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹⁰

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹², specifically:

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ¹¹

This international Searching Authority found multiple inventions in this international application as follows:

Claims 1-4 drawn to a peptide

Claims 5-6 drawn to DNA, RNA, and microorganism

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☒ No protest accompanied the payment of additional search fees.

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